Stratum corneum activation of complement through the antibody-independent alternative pathway

博士論文

Stratum Corneum Activation of Complement Through the Antibody-independent Alternative Pathway

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ABSTRACT

We provide evidence that stratum corneum (SC) activates complement through the alternative pathway to produce C5a anaphylatoxin. By immunofluorescence study it was shown that in addition to circulating IgG autoantibody against the SC, there were anti-SC antibodies of IgM and IgA classes in the sera from normal individuals. However, all the titers were significantly lower than the level of C3 deposition between corneocytes. By contrast, there occurred no C1q deposition.

Immnoelctrophoretically the orthokeratotic SC homogenates were found to induce the conversion of C3 from native C3 to C3b in fresh human serum even when the classic pathway was blocked by Ca^{2+}-chelation. Enzyme immunoassay showed that factor B split product, Bb, was generated by the SC homogenates in the Ca^{2+}-chelated serum. Radioimmuoassay for C5a also demonstrated that the SC homogenates could generate C5a anaphylatoxin in serum to an extent similar to that noted in non-treated serum when restricted to the alternative activation; neutrophil chemotactic activity was generated in Ca^{2+}-chelated serum at levels comparable to that generated in non-treated fresh serum. We separated the SC samples into the cornified envelope and keratin fractions. The cornified envelope fraction was more effective in activating complement. This activity resided in heat-stable and non-lipid substances of corneocytes. Our hypothesis is that, when the SC comes in contact with serum, it activates complement mainly through the alternative pathway to induce chemotactic C5a anaphylatoxin. Hence, inflammation in normal individuals after a traumatic injury to the skin or rupture of acne or epidermal cysts and possibly the formation of subcorneal sterile pustules noted in several dermatoses are explainable through this mechanism.

INTRODUCTION

Stratum corneum (SC) has multiple protective functions against chemical and mechanical insults (1). It is the major barrier to diffusion of potentially toxic substances. Despite these important functions, it is known that a direct exposure of the SC to the living tissues induces intense inflammatory changes (2). There are also several dermatoses characterized by sterile pustule formation at the subcorneal location such as pustulosis palmaris et plantaris (PPP) and subcorneal pustular dermatosis in which we often find detached fragments of the SC densely surrounded by many neutrophils. Anti-SC antibodies are demonstrable in all human sera (3) and they have been implicated in the pathogenesis of psoriasis and the above mentioned dermatoses in which subcorneal pustules form as a result of transepidermal leukocyte chemotaxis from inflamed blood vessels in lesions of psoriasis (4-6).

Danno et al. (7) found higher titers of anti-SC antibodies in patients with PPP both in IgG and complement-fixing antibodies. In contrast, Tagami et al. (8) did not found higher titers of circulating anti-SC IgG antibodies or complement-fixing anti-SC antibodies in psoriatic patients. Therefore, there is another possibility that the antibody-independent alternative pathway may be involved in complement activation by the SC.

In the present study, we found that human sera contain anti-SC antibodies other than IgG class such as IgM and IgA classes. However, we have shown that the SC activates complement through the alternative pathway to generate C5a anaphylatoxin. This activity mainly resides in the cornified envelope faction of corneocytes as heat-stable and non-lipid substances.

MATERIAL AND METHODS

Skin samples: Normal human skin sample was obtained from the buttock of a 34-year-old male. The skin was frozen immediately and used as a substrate for indirect IF.

Normal human SC samples were obtained from the sole of five healthy individuals aseptically as described by Dalziel et al. (2). To prevent bacterial contamination the skin was cleaned first with isopropyl alcohol. The first scrapings where bacteriae live (9) were discarded with a sterile scalpel blade. The remaining SC was pared off and kept frozen at -80°C. Their bacteriological and mycological studies yielded negative results.

Preparation of SC homogenates: The SC samples were dried in the desiccator, then crushed in a Freezer/Mill (Spex Industries, Metuchen, NJ, USA). Thereafter they were suspended in phosphate-buffered saline (PBS) pH 7.4 at 50 times the original dry weight, and homogenized with a Potter-Elvehjem device. The homogenates were stirred at 4°C for 24 hours and centrifuged at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in the original volume of PBS. This suspensions were used for C5a anaphylatoxin radioimmunoassay and neutrophil chemotactic assay . To exclude bacterial lipopolysaccharide (LPS), a potent activator of the alternative complement pathway, we carried out Lymulus lysate tests (10) using Pyrogent (Whittsaker M.A. Bioproducts, Walkersville, MD, USA), the sensitivity of which is greater than 0.25 EU/ml. The SC homogenate samples were uniformly negative.

Separation of SC into the cornified envelope and soluble and keratin fractions: We followed the procedure described by Nagae et al. (11). Briefly the SC samples were washed three times with PBS, suspended in a small amount of 2% sodium dodecyl sulfate (SDS), 20 mM 2-mercaptoethanol (2-ME), and heated for 10 min in a 100°C water bath. As a result, we obtained as an insoluble detergent- and reducing agent-resistant cornified envelope fraction as well as a soluble fraction

containing keratin polypeptides and other soluble components; we call the latter as the keratin fraction hereafter. These were dialyzed and lyophilyzed. For complement activation assay they were resuspended in PBS at 50 times the original dry weight.

Extraction of lipid from stratum corneum homogenates: The lipid containing in the SC homogenates were extracted successively with 10 ml portions of chloroform:methanol, 2:1, 1:1, and then 1:2, at a room temperature for a period of 2 hours, respectively. The resultant lipid-free SC homogenates were dried under a stream of nitrogen. Then neutrophil chemotactic activity was compared between the lipid-extracted and the non-extracted SC homogenates.

Serum samples: Blood samples were collected from 10 healthy adults without any skin diseases. Separated fresh sera (non-treated serum: NTS) were stored at -80^{o}C until use.

Aliquots of the sera were depleted of complement activity by heat (56^{o}C for 30 min; heat-inactivated serum: HIS). The serum was chelated by the addition of 1/5 volume of 0.1 M ethylenediamine tetraacetic acid (EDTA) to prevent any complement activation , or by 0.1 M ethyleneglycol-bis (β-amino-ethylether)-N,N'-tetraacetic acid (EGTA) supplemented with 10 mM Mg^{2+} in 5 mM veronal buffer (Ca^{2+}-chelated serum: CaCS), that permits only the alternative pathway activation, but not the classic pathway activation. One volume of the SC homogenates was added to 5 volumes of pretreated or nontreated serum.

Immunofluorescence: Titers of anti-SC autoantibodies were determined in normal skin by the standard indirect IF method using FITC-labeled antibodies against human IgG, IgA, and IgM. Indirect IF staining for complement was performed by allowing to fix at the same time as the autoantibodies were bound to the SC antigen and by treating with labeled anti-C3 and anti-C1q conjugates as described before (12). NTS and CaCS were used without heat inactivation.

The characteristics of fluorescein-conjugated sera (Behring Institute, Germany) were as follows: i) anti-human IgG (Lot No.128811A)-molar FITC/protein ratio (F/P ratio), 2.1; ii) anti-human IgA (Lot No.128512B)-F/P ratio, 2.6; iii) anti-human IgM (Lot No.128414)-F/P ratio, 2.9; iv) anti-human C1q (Lot No.129315A)-F/P ratio, 2.5.

Undiluted or diluted sera were placed on frozen skin sections that had been washed beforehand in PBS, and incubated at 37°C for 30 min in a moist chamber. They were then washed in PBS and incubated again at 37°C for 30 min in the moist chamber with a 1:40 or 1:80 (only anti-human IgG) dilution of the above conjugates. After washing in PBS, the stained sections were examined under a immunofluorescence microscopy (Vanox, Olympus, Japan). The reciprocal of the dilution just before negative staining was regarded as the titer of anti-SC autoantibodies.

Direct IF staining of each substrate with these conjugates was conducted simultaneously to exclude false-positive reactions due to anti-SC antibodies contained in the conjugates. In all cases, the conjugates were negative for staining.

Average logarithmic titers ($\log_{10}$) were analyzed in pairs by Student's t-test. Each titer was also compared using a Spearman rank correlation coefficient between different classes of antibodies. Negative results were assumed to have a value of one.

Immunoelectrophoresis: Immunoelectrophoresis for C3 was performed on glass plates using 1% agarose (Agarose I; Wako Pure Chemical, Japan) in veronal buffer (pH 8.6; ionic strength, 0.05) with a continuous potential gradient of 2 mA/cm for 4 h at 4°C. Goat antisera to human C3 (Miles Lab., West Haven, CT, USA) was poured into the troughs and test sera into the wells.

Enzyme immunoassay for factor B split product, Bb: Enzyme immunoassay was performed by using an assay kit for Bb, which was a gift from Cytotech (San Diego, CA, USA).

Radioimmunoassay for C5a and C5a des arg: Radioimmunoassay for C5a and C5a des arg was performed by using an assay kit for C5a purchased from Upjohn Diagnostics (Kalamazoo, MI, USA). The methodology of this kit is selective for C5a and C5a des arg, eliminating assay interference from C5.

Neutrophil chemotaxis assay: Fresh serum obtained from normal individuals was incubated with the SC at 300 times the original weight for one hour, before or after each serum treatment, and then incubated at 56^{o}C for 30 min. As a control, an aliquot of the serum was simply incubated at 37^{o}C for one hour and later heat-inactivated in the same way. After centrifugation at 3000 rpm for 10 min the supernatant was diluted 10 times with PBS and assayed for neutrophil chemotactic activity. All the samples were tested in triplicate and the results were expressed as means $\pm$ standard deviations (SD). Neutrophil chemotaxis was measured in a modified Boyden chamber assay with 3 micrometer Millipore filter as described before (13).

RESULTS

Indirect IF: Nine of 10 serum samples from normal individuals showed a positive linear IgG staining between the corneocytes of normal skin when the sections were treated with undiluted sera. The titers of IgG autoantibodies ranged from negative to 80. Nearly all of the sera displayed positive intercellular IgA and IgM staining of the SC. The titer of IgA autoantibodies ranged from negative to 40, and those of IgM autoantibodies from 10 to 40 as shown in Table I-A. They were lower than that of IgG antibody (IgM vs IgG, no significant difference; IgA vs IgG, $p < 0.005$). Despite these results for anti-SC antibodies, no C1q deposition was noted with any of the serum samples obtained from normal individuals (Table I-B).

In contrast to C1q, the IF reaction pattern for C3 with undiluted sera was so bright that corneocytes appeared to be stained homogeneously. The characteristic linear or dot-like intercellular deposits of C3 became discernible only with higher dilutions. The specificities of the positive staining reactions was confirmed by negative results with HIS. These titers ranged from 20 to 160 (Table I-B). The average titer for C3 was significantly higher than that for IgG ($p < 0.005$), IgM ($p < 0.001$), or IgA ($p < 0.001$). In addition, there was no correlation between the titers for C3 deposition and those of IgG, IgA, or IgM autoantibodies (data not shown), suggesting that C3 deposition takes place irrelevant to anti-SC autoantibodies.

We conducted the same indirect complement IF tests using CaCS instead of NTS. Although positive intercellular C3 staining of the SC was noted with CaCS, the average titer was lower than that of NTS (Table I-B).

Immunoelectrophoresis: The incubation (60 min) of the SC homogenates with non-treated normal human serum (NTS) resulted in the conversion of C3 from native C3 to C3b, but such extra precipitation peak was absent from the Ca^{2+}- and Mg^{2+}-free serum resulted from the treatment with EDTA, in which both the classic and alternative pathways were blocked. On the other hand, the same C3 conversion was observed in CaCS, where only the alternative pathway is allowed to be activated (Fig. 1).

Bb fragment production by SC homogenates: As shown in Fig. 2, Bb fragment generated in both NTS and CaCS by incubation with the SC homogenates for 60 min. The generated amount of Bb was not significantly reduced by Ca^{2+}-chelation. This observation confirms that the SC activates complement through the alternative pathway.

C5a production by SC homogenates: In the previous experiments, we indicated that the alternative complement pathway can be activated by the SC. The next experiments was designed to ask if the SC homogenates can produce C5a anaphylatoxin through the complement activation. The results (Table II) showed that C5a and C5a des arg were generated in NTS by incubation with the SC homogenates for 60 min. No such production of C5 cleavage products was observed with HIS. The generation, however, was unaffected even when the classic pathway was selectively blocked by Ca^{2+}-chelation (Table II).

Generation of neutrophil chemotactic activity after activation of complement by SC: We examined neutrophil chemotactic activity in sera obtained in the same way as shown in Table II. The orthokeratotic SC samples produced chemotactic activity for neutrophils in NTS after incubation at 37°C for one hour. Moreover, the chemotactic activity generated by the SC in CaCS was almost comparable to that noted in NTS (Fig. 3). The SC samples did not generate chemotactic activity in HIS (data not shown).

Generation of neutrophil chemotactic activity by the cornified envelope and keratin fractions from SC: The SC was separated into the cornified envelope and keratin fractions to investigate the structural component of the SC responsible for the alternative pathway activation. Both the fractions produced chemotactic activity for

neutrophils in NTS, the former giving higher values. The chelation of Ca^{2+} did not abrogate the generation of chemotactic activity induced by them (Fig. 4).

Effects of heat- or lipid solvent-treatment of SC homogenates on the generation of neutrophil chemotactic activity in fresh serum: The next experiment was carried out to further characterize substances in the SC to activate complement. As shown in Table III, the heat-treated (at 100°C for 5 min) SC homogenates produced neutrophil chemotactic activity in NTS at a level comparable with that noted with the non-treated SC homogenates. Lipid extraction from the SC homogenates did not affect the generated neutrophil chemotactic activity. These results suggest that heat-stable and non-lipid substances of corneocytes were responsible for the complement activation.

DISCUSSION

Our present indirect IF study in frozen sections of normal skin demonstrated that there was coexistence of SC autoantibodies of IgG, IgA, and IgM classes in sera of normal subjects. The titers for C3 deposition in the SC significantly higher than those of these autoantibodies in most cases, but deposition of C1q in the SC was not found at all, although the lack of demonstration of C1q does not necessarily mean that it is absent. These results indicate that the complement activation by the SC does not occur through the classic pathway alone. Complement activation by the SC occurred in CaCS suggests that both the classic and alternative pathways can be activated by the SC with a resultant higher titer for C3 deposition in the SC than those for immunoglobulines or C1q demonstrated by the IF method.

Immunoelectrophoretically the orthokeratotic SC homogenates were found to be able to induce the conversion of C3 from native C3 to C3b in fresh serum even when the classic pathway was blocked by Ca^{2+}-chelation. The results of enzyme immunoassay for the generation of Bb also confirmed that the SC homogenates activate the alternative complement pathway. Our *in vitro* experiments for the production of C5a by the orthokeratotic SC homogenates showed that C5a could be produced in CaCS at a level similar to that in fresh serum, i.e. the C5a generation was not affected by the selective blockage of the classic pathway by Ca^{2+}-chelation. Furthermore, we found that the neutrophil chemotactic activity induced in CaCS was comparable to that noted in NTS. Although we were unable to carry out experiments using the serum samples deficient in the early complement components such as C1, C4, or C2, all of these findings strongly would suggest that the alternative pathway activation is dominant for the production of chemotactic C5a. They are also interesting because Iwatsuki et al. (14) found only a decrease of C3 receptors but not of IgG-Fc cell surface receptors of neutrophils obtained from pustular lesions of psoriasis and PPP, suggesting the predominance of the alternative pathway activation in the formation of subcorneal pustule in these dermatoses. These results support the concept that complement

activation occurs through the antibody-independent alternative pathway in pathological conditions characterized by sterile subcorneal pustule formation.

In an effort to identify the structural component of the SC responsible for the alternative complement activation, we separated the SC samples into the cornified envelope and keratin fractions. We found that the fraction of cornified envelopes, which enclose keratin filaments in the horny layer, produced chemotactic activity more efficiently than the keratin fraction even in CaCS. Further study showed that heat-stable, non-lipid substances of corneocytes were responsible for the alternative pathway activation.

The exact mechanism of SC activation of alternative pathway is not clear. At lease there are two possibilities: 1) contamination of bacterial LPS or fungi and 2) the absence of sialic acid in the SC. The possibility of bacterial LPS contamination was ruled out by the negative Lymulus lysate test. The possibility of the presence of yeast flora is also remote because the C3 deposition occurred at the intercellular position of deep portion of the SC in indirect IF and because the used SC samples were found to be sterile by culture. Therefore, at present we believe that the second possibility is the most plausible. It has been observed in many systems (15-18) that the presence or absence of surface sialic acid influences on the alternative pathway activation. In the case of the epidermis, keratinocyte membrane sugars display a vectorial pattern of progressive, sequential sugar additions to the cell membrane during differentiation to the level of the granular layer. Virtually all such lectin labelling abruptly disappears from the SC cell membrane, suggesting that corneocytes lack sialic acid on their surfaces (19-22). This disappearance of sialic acid in the SC seems to play a pivotal role in the activation of the alternative complement pathway.

The possibility of the existence of some protease(s) in the SC that directly splits C5 to produce C5a anaphylatoxin could not be directly denied from our present experiments. However, the result that heating of the SC did not significantly influence the generation of neutorophil chemotactic activity suggests that heat-labile high molecular proteins such as proteases may not responsible for this complement

activation. Also, Lazarus et al. (23) found that the protease activity that was inhibited by diisopropyl fluorophosphate was much lower in psoriatic uninvolved skin than that of the psoriatic plaque. Hence, it is reasonable to assume that the protease activity in normal SC is very low.

Based on our findings we think that, when the SC, especially its cell surfaces, comes in contact with plasma or serum, the complement system becomes activated mainly through the alternative pathway to produce chemotactic C5a anaphylatoxin. This may account for the inflammation in normal individuals after a traumatic injury to the skin and especially after rupture of epidermal cysts (24) or acne comedones and possibly for the formation of subcorneal pustules noted in several dermatoses, including psoriasis, the pathogenesis of which is known to be associated with abnormal cellular immune reactions (25-27). With regard to psoriasis, we assume that complement activation and neutrophil accumulation are responsible for completion of the formation of typical psoriatic lesions, since we could find neutrophil accumulation in typical psoriatic plaque, but not in the initial pinpoint lesion of active psoriatics (25). Recently, other neutrophil chemotactic factors including IL-8 has been reported. In our laboratory it has been indicated that C5a anaphylatoxin play a more significant role in the neutrophil chemotaxis by psoriatic scale extracts than IL-8 (Submitted data).

In summary, we found that human sera contain anti-SC antibodies other than IgG class such as IgM and IgA classes. However, we have shown that the SC activates complement through the alternative pathway to generate C5a anaphylatoxin. This activity mainly resides in the cornified envelope faction of corneocytes as heat-stable and non-lipid substances.

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LEGENDS FOR FIGURES

Fig. 1 - Immunoelectrophoresis using goat anti-human C3. The troughs contain anti-human C3 antisera, and the wells contain test sera. Upper, non-treated serum was incubated with the SC homogenates; Middle, serum treated with EDTA; Lower, serum treated with Mg^{2+}-supplemented EGTA.

Fig. 2 - The results of enzyme immunoassay for factor B split product, Bb, produced by SC homogenates. Bb was generated by the SC homogenates in CaCS as well as in NTS. The amount of Bb fragment was not significantly reduced by Ca^{2+}-chelation. NTS: non-treated serum; CaCS: Ca^{2+}-chelated serum; HIS: heat inactivated serum; ZAS: zymosan activated serum (1:10 dilution).

Fig. 3 - Neutrophil chemotactic activity after the complement activation by SC homogenates. The chemotactic activity generated by the SC homogenates in CaCS was compared to that noted in NTS.

Fig. 4 - Neutrophil chemotactic activity by activation of complement with the cornified envelope and keratin fractions obtained from SC. The cornified envelope fraction as well as the keratin fraction produced chemotactic activity but that generated by the former was higher than that by the latter.

Table I. Immunofluorescence study of complement activation on stratum corneum of cryo-section with sera of normal subjects.

A. Titers of anti-stratum corneum auto-antibody

Immunoglobulins	serum treatment	titers (average in logarithm) n=10	
IgG	NTS[a]	0 to 80	(1.26 ± 0.29)
IgM	NTS	0 to 40	(1.08 ± 1.17)
IgA	NTS	10 to 40	(0.72 ±0.38)

B. Titers of complement component deposition

Complements	serum treatment	titers (average in logarithm) n=10	
C3	NTS	20 to 160	(1.78 ± 0.08)
C3	CaCS[b]	10 to 80	(0.85 ± 0.58)
C1q	NTS	0	

[a]NTS: non-treated serum.

[b]CaCS: Ca^{2+}-chelated serum.

Table II. Radioimmunoassay for C5a and C5a des arg produced by orthokeratotic stratum corneum.

	C5a and C5a des arg (ng/ml)			
	PBS[a]	NTS[b]	HIS[c]	CaCS[d]
orthokeratotic SC homogenates mean ± SD (n=6)	8 ± 4	145 ± 14	11 ± 2[e]	160 ± 37

[a]PBS: phosphate-buffered saline.

[b]NTS: non-treated serum.

[c]HIS: heat inactivated serum.

[d]CaCS: Ca^{2+}-chelated serum.

[e]: C5a production was significantly less than that noted in NTS ($p < 0.001$).

Table III. Effects of heat- or solvent-treatment of SC on the generation of neutrophil chemotactic activity in human fresh serum.

skin	treatment of stratum corneum		serum	PMN/HPF[c]
sample	boiling[a]	lipid extraction[b]		
(+)	(-)	(-)	NTS[d]	380 ± 58
(+)	(+)	(-)	NTS	281 ± 39
(+)	(-)	(+)	NTS	352 ± 22
(+)	(-)	(-)	(-)	11 ± 5
(-)	(-)	(-)	NTS	99 ± 49

[a]: boiled at 100°C for 5 min.

[b]: Lipid was extracted from the SC as described in the Materials and Methods section.

[c]PMN/HPF: polymorphonuclear leukocyte per one high power field (x400).

[d]NTS: non-treated serum.

Figure 1.

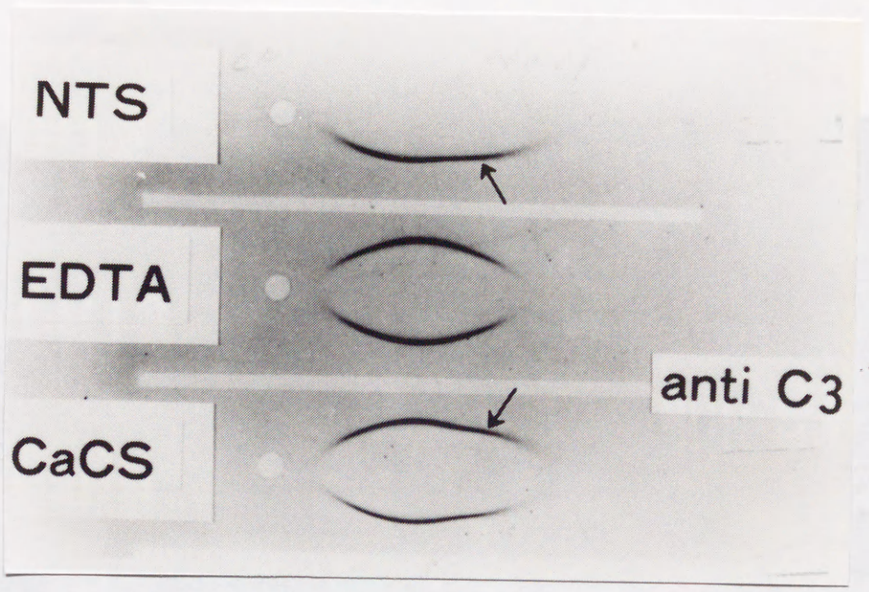

Figure 2.

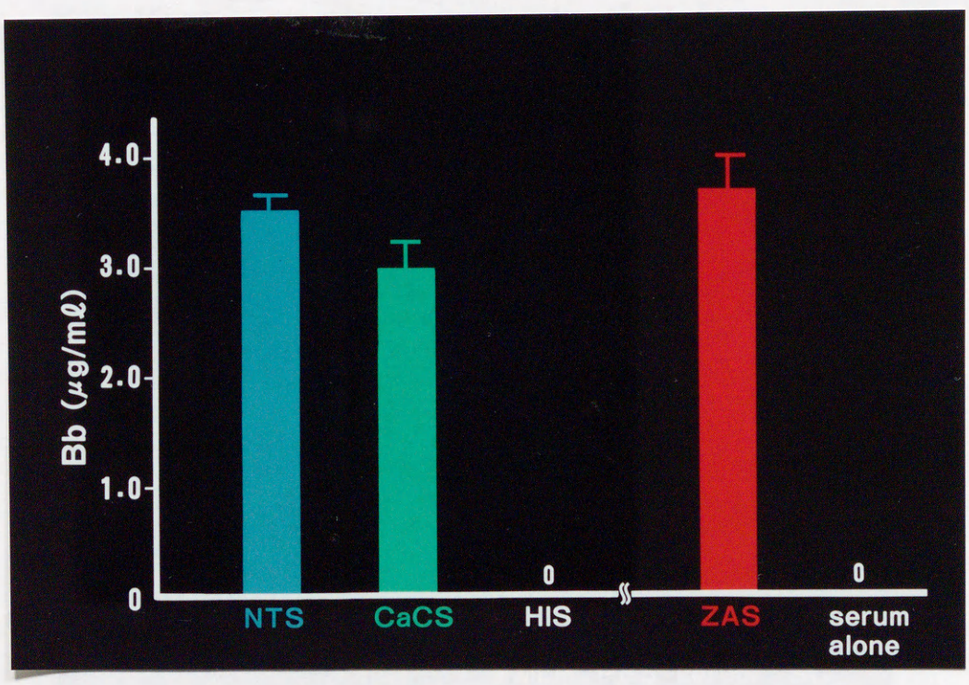

Figure 3.

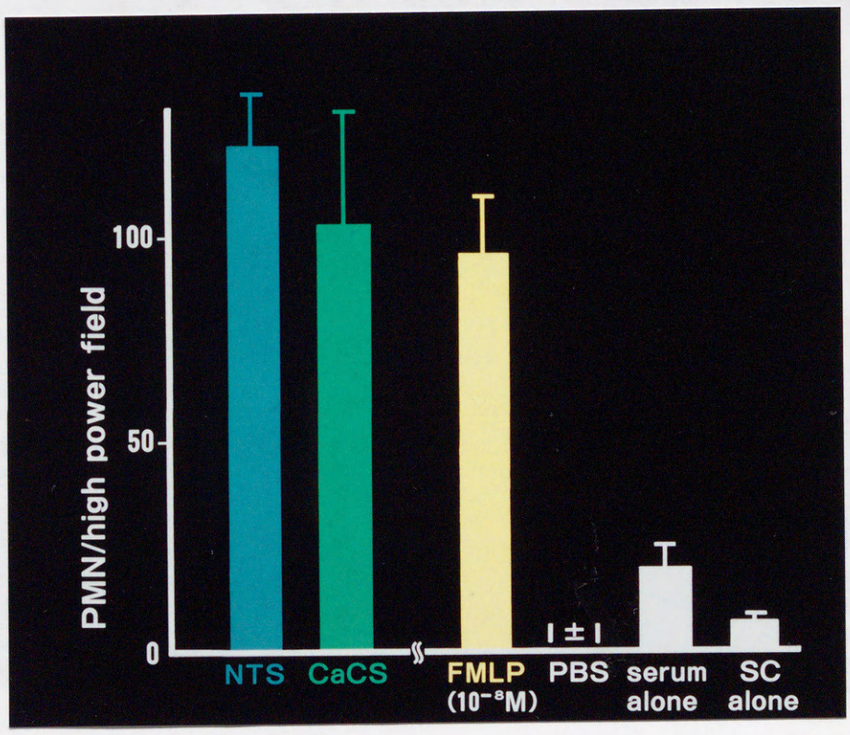
PMN/high power field
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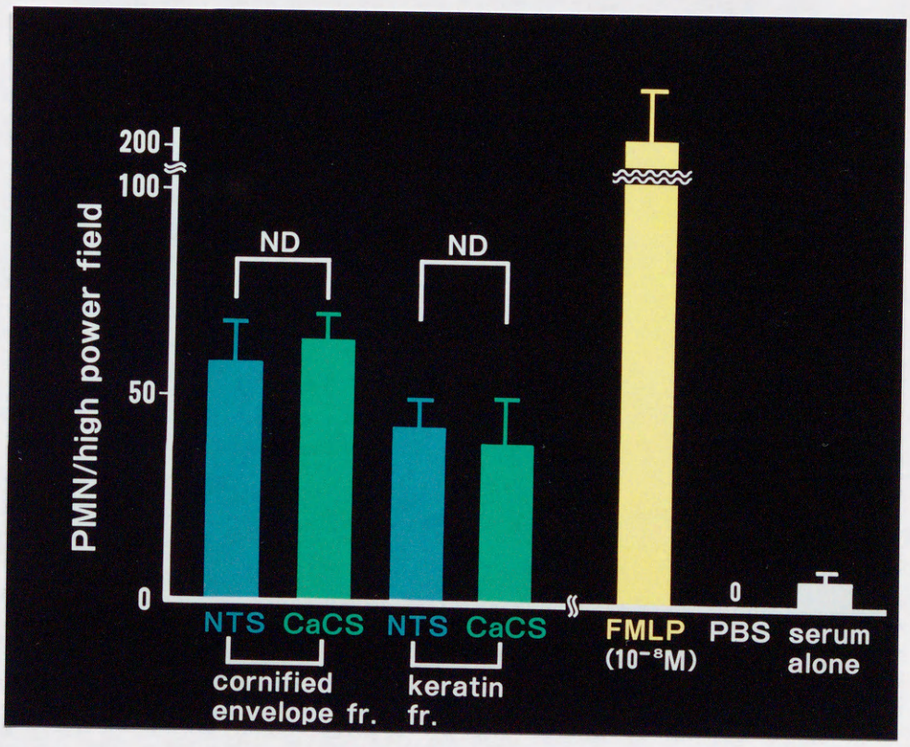
Figure 4.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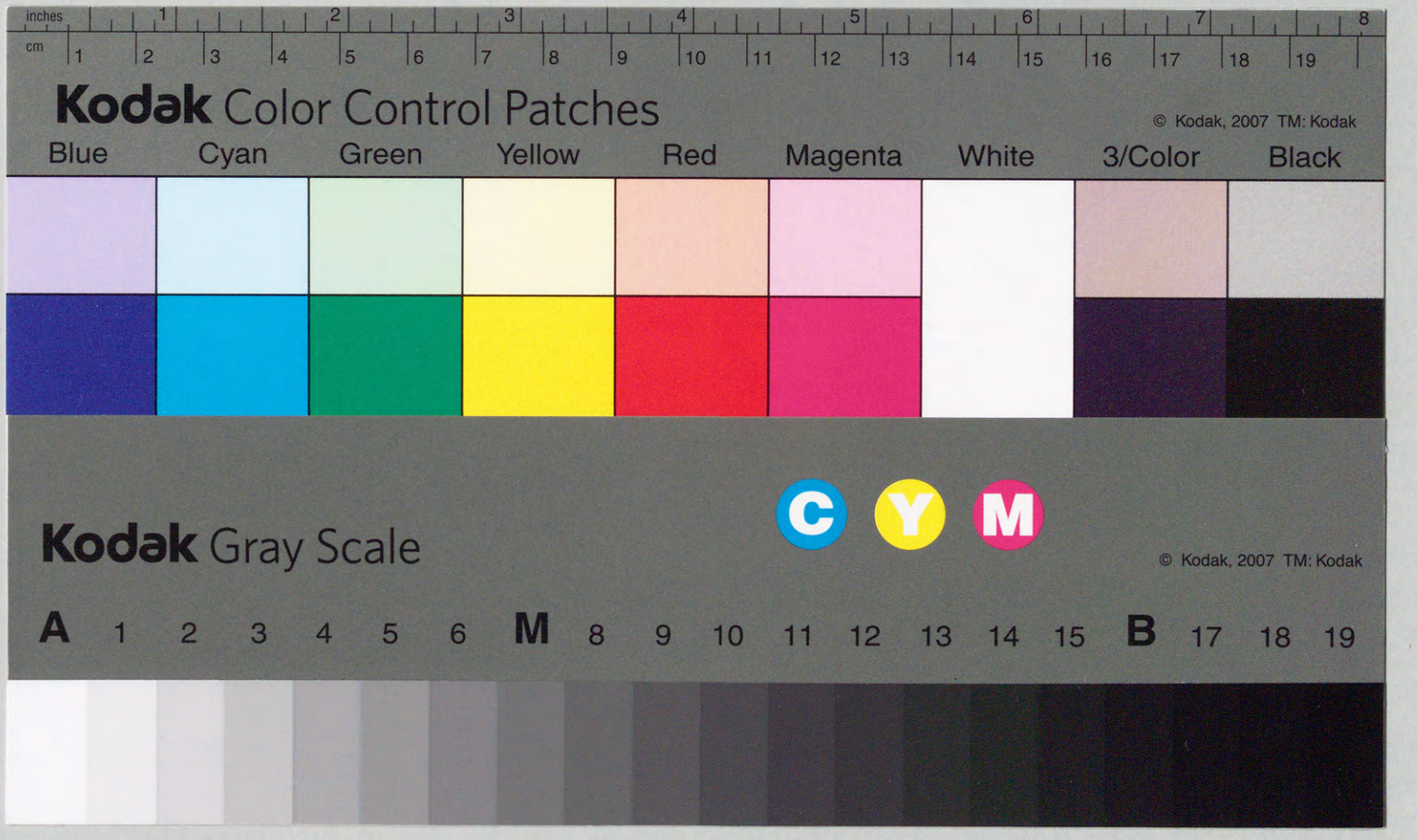
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